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*J Immunol* 2007; 178:7767-7778;
doi: 10.4049/jimmunol.178.12.7767
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The positive regulation of the NF-κB-signaling pathway in response to TCR stimulation has been well-studied. However, little is known about the negative regulation of this pathway in T cells. This negative regulation is crucial in controlling the duration of TCR signaling and preventing abnormal lymphocyte activation and proliferation. Therefore, understanding the negative regulation of TCR-mediated NF-κB signaling is essential in understanding the mechanisms involved in T cell function and homeostasis. TCR stimulation of human CD4+ T cells resulted in an increase in NF-κB2/p100 expression with no appreciable increase in p52, its cleavage product. Due to the presence of inhibitory ankyrin repeats in the unprocessed p100, this observation suggests that p100 may function as a negative regulator of the NF-κB pathway. Consistent with this hypothesis, ectopic expression of p100 inhibited TCR-mediated NF-κB activity and IL-2 production in Jurkat T cells. Conversely, knockdown of p100 expression enhanced NF-κB transcriptional activity and IL-2 production upon TCR activation. p100 inhibited the pathway by binding and sequestering Rel transcription factors in the cytoplasm without affecting the activity of the upstream IκB kinase. The kinetics and IκB kinase γ/NF-κB essential modulator dependency of p100 induction suggest that NF-κB2/p100 acts as a late-acting negative-feedback signaling molecule in the TCR-mediated NF-κB pathway. The Journal of Immunology, 2007, 178: 7767–7778.

The CD4+ Th cell is involved in the immune response against pathogenic challenges by activating other immune cells such as B cells and macrophages. These responses are mediated by the TCR, which is able to recognize Ag-derived peptides presented by MHC II glycoproteins on APCs. The TCR is composed of an α- and β-chain on the cell surface, and the CD3 complex, which transduces signals across the cell membrane. The cytosolic portion of the CD3 complex is subject to phosphorylation by the src-family of protein tyrosine kinases (PTK)1 Lck and Fyn (1). This recruits Zap70, a member of the SYK family of PTKs, to the complex, and once activated it is able to phosphorylate other proteins involved in TCR signaling, such as linker of activated T cells, Grb2-like adaptor downstream of Shc, and phospholipase Cγ1 (1–4). Upon activation, phospholipase Cγ1 hydrolyzes phosphoinositides to inositol-1,4,5-trisphosphate and diacylglycerol, which are involved in the elevation of intracellular calcium and activation of protein kinase C (PKCε), respectively (1). The increase in intracellular calcium then leads to the activation of calcineurin, a serine/threonine phosphatase that dephosphorylates the transcription factor NFAT, thus allowing it to translocate to the nucleus to regulate gene expression (5).

T cell activation also requires a secondary signal from a costimulatory receptor, such as the CD28 coreceptor (6). Studies have shown that CD28 engagement activates a number of effectors, such as ITK, LCK, VAV-1, PI3K, and AKT, which will aid in the proximal TCR-signaling machinery (7). In addition to regulating proximal TCR signaling, CD28 costimulation enhances and positively regulates TCR-mediated signaling distally in that it can enhance the phosphorylation and degradation of IκBα, leading to the release of NF-κB transcription factors (8). In mammals, there are five NF-κB transcription factors that can form either homodimers or heterodimers: RelA (p65), RelB, cRel, NF-κB1 (p105), and NF-κB2 (p100). In the canonical NF-κB pathway, RelA/p50 heterodimers are sequestered in the cytoplasm by a family of IκBs (9). The degradation of IκBα is initiated by activation of the IκB kinase (IKK) complex, which is composed of three subunits: IKKe and IKKβ are serine/threonine kinases and serve as catalytic subunits and IKKγ (NF-κB essential modulator (NEMO)) is the regulatory subunit that interacts with IKKe and IKKβ (9). Upon phosphorylation, IκBα is ubiquitinated and thus tagged for degradation by the 26S proteasome complex (9). This allows the NF-κB dimer to translocate to the nucleus where it regulates the transcription of genes involved in inflammation, immune processes, apoptosis, and cancer.

Whereas RelA, RelB, and cRel are synthesized as mature proteins containing a transcriptional transactivation domain and a DNA-binding Rel homology domain, NF-κB1 (p105) and NF-κB2 (p100) are processed into p50 and p52, respectively, from the full-length products of the NFKB1 and NFKB2 genes. This processing is important because the p105 and p100 precursors contain IκB-like ankyrin repeats in their C termini, and so they can function as IκBs (10, 11). Whereas the cleavage of NF-κB1/p105 to p50 occurs constitutively, that of NF-κB2/p100 to p52 occurs in a regulated manner (12, 13). In the latter, newly synthesized p100 forms inactive heterodimers with RelA, RelB, or cRel in the cytoplasm and upon activation of the noncanonical NF-κB pathway, NF-κB-inducing kinase (NIK) phosphorylates IKKe, which in turn phosphorylates p100 (13, 14). This leads to the ubiquitination and degradation of the IκB-like C terminus of p100, allowing the resultant p52/rel heterodimer to translocate to the nucleus. Thus, NF-κB2/p100 has the ability to participate in NF-κB transcriptional activity via the regulated processing and generation of an active p52 subunit. In addition to transcriptional activation, the presence of the IκB-like ankyrin repeats in the C terminus of p100 suggests that it...
also has the ability to retain NF-xB in the cytoplasm (10). Indeed, it has been shown that p100 is capable of binding to RelB to inhibit its nuclear translocation and RelB-dependent transcriptional activity (15, 16). Several groups have recently demonstrated that p100 can function as a negative regulator of biological processes involving NF-xB signaling. One group suggested that p100 can control osteoclastogenesis via its inhibitory property (17). In osteoclast precursors, the stimulation of the NF-xB-signaling pathway by the cytokine receptor activator of NF-xB ligand, which is necessary for osteoclast differentiation, induced the expression of p100 and its processing to p52 (17). Osteoclast precursors from NIK-deficient mice exhibited an accumulation of p100 in the cytoplasm, an inability to process p100 to p52, and a decrease in NF-xB-binding activity in the nuclei, suggesting that the accumulation of unprocessed p100 prevented the translocation of NF-xB complexes to the nucleus (17). Similarly, memory T cells from NIK-deficient mice were reported to have impaired IL-2 production in response to TCR ligation due to the accumulation of unprocessed p100 (18). These studies using NIK-deficient mice suggested the possibility that p100 may function as a negative regulator during T cell activation. However, because the possibility exists that NIK may regulate targets other than p100 and a direct examination of the role of p100 in TCR signaling has not been conducted, its function during this process remains to be fully clarified.

Like most complex biological systems, the TCR-signaling pathway is controlled by both positive and negative regulation. The initiation and transmission of TCR signaling has been extensively studied, as discussed previously. In addition, the signaling pathway at the level of IKK is beginning to be clarified. PKCθ was shown to associate with CARMA1/CARD11, which is necessary for NF-xB activation in response to TCR/CD28 activation (19). Along with Bcl10 and CARMA1, which can associate with each other (20), PKCθ and IKKβ assemble at the immunological synapse upon TCR/CD28 activation (21). Bcl10 also associates with MALT1, a ubiquitin E3 ligase, thus initiating lysine 63-type ubiquitination of NEMO (22). Gene-targeting studies have shown that TCR/CD28-induced NF-xB activation in Bcl10-/-and MALT1-/-T cells is impaired (22, 23). RIP2 also associates with Bcl10, and mice that are deficient in RIP2 were also defective in TCR/CD28-induced NF-xB activation (24).

In contrast to the well-studied positive regulation of TCR signaling, less is known about the down-regulation and termination of this process. Studies have shown that the CD28 coreceptor enhances TCR signaling and without it TCR ligation leads to T cell anergy, which is characterized by a state of unresponsiveness (25). The ubiquitin ligases c-Cbl and Cbl-b were shown to remove the TCR complex from the cell surface, thus halting TCR signaling (26). In addition, it has been postulated that TCR engagement activates some protein phosphatases that counterbalance the effects of the PTKs that initiate T cell activation (27). After being recruited to the immunological synapse, Bcl10 has been shown to be phosphorylated and subject to ubiquitination, thus leading to its degradation and cessation of the TCR/CD28-induced NF-xB activation (28). At the level of NF-xB, the inhibitory protein IκBα that sequesters the NF-xB dimer in the cytoplasm is itself an immediate target of NF-xB-mediated gene transcription (29). This rapid induction of IκBα re-expression provides a negative-feedback mechanism leading to the separation of nuclear NF-xB from its DNA-binding site and transport back out to the cytoplasm (29). The negative regulation of T cell signaling is critical in maintaining T cell homeostasis, controlling the duration of TCR signaling, and preventing abnormal lymphocyte activation (27).

In this study, the role of NF-xB2/p100 in TCR signal transduction was examined and the results suggest that p100 may function in a later negative-feedback manner to down regulate NF-xB signaling.

Materials and Methods
Plasmids and reagents
NF-xB2/p100 and p100ΔGRR constructs were gifts from Dr. W. Greene (University of California, San Francisco, CA) and Dr. S.-C. Sun (Pennsylvania State University College of Medicine, Hershey, PA). NF-xB2/p100 and p100ΔGRR were amplified by PFU Turbo DNA polymerase (Stratagene) and cloned into a retroviral vector upstream of an internal ribosomal entry site-puromycin-resistance cassette (30). For some experiments, a retroviral construct containing a FLAG-tag at the N terminus of NF-xB2/p100 was used. Retroviral transduction of Jurkat T cells was performed as previously described (30). Bulk population of drug-resistant cells was used in experiments to avoid clonal variability. For transient expression, NF-xB2/p100 was transfected by electroporation using a Bio-Rad Gene Pulser Electroporator set at 250 V, 975 μF. Bacterial GST cloned into retroviral vector was used as a negative control. The NF-xB luciferase reporter construct contains multipliers of the NF-xB consensus sequence upstream of luciferase (31). RE/AP luciferase reporter construct, which was a gift from Dr. A. Weiss (University of California, San Francisco, CA), contains the CD28 responsive element (CD28RE) and the NFIL-2B AP-1 site upstream of luciferase (32–34). PMA and ionomycin were obtained from Sigma-Aldrich. Anti-CD3 Ab (OKT3) was obtained from Ortho Biotech. Staphylococcal enterotoxins D (SED) and B (SEB) were obtained from Toxin Technology.

Cell cultures
The Jurkat T cell line and the IKK-y-deficient cell line have been described previously (30). Raji B cells were a gift from Dr. P. Leibson (Mayo Clinic College of Medicine, Rochester, MN). Cells were maintained in IMDM medium (Sigma-Aldrich) supplemented with 10% heat-inactivated bovine calf serum (HyClone), 50 mM 2-ME (Sigma-Aldrich), 2 mM L-glutamine (Cellgro), and 15 μg/ml gentamicin. Raji B cells were fixed in 0.5% paraformaldehyde for 10 min and washed three times in serum-free IMDM. PBMC from human buffy coat were enriched by SRBC rosetting. Human CD4+ T cells were obtained from PBMCs using the Naive CD4+ T cell Isolation kit (Miltenyi Biotec) according to the manufacturer’s instructions.

Small-interfering RNA (siRNA)
siRNA-mediated gene silencing was performed by using the retroviral expression vector pSUPERretro (Oligoengine) to express small hairpin RNA (shRNA). The NF-xB2/p100-specific insert consisted of a 19-nt sequence (GATTGAGCGGCCTGTAACA) separated by a noncomplementary spacer from the reverse complement of the same 19-nt sequence to form the shRNA duplex, referred to as pSR si-p100. A nonsilencing (pSR si-NES) control vector (TTCTCCGAACGTGTCACGT) was also designed in this manner. Transient knockdown of p100 in Jurkat T cells was achieved by electroporation of pSR si-NES or pSR si-p100 using a Bio-Rad Gene Pulser Electroporator set at 250 V, 975 μF. For stable knockdown, these constructs were modified so that the puromycin-resistance cassette was replaced by GFP. These constructs are referred to as pSR-GFP si-p100 and pSR-GFP si-NES. Retroviruses generated from these constructs were used to infect Jurkat T cells as previously described (30). Selection for retrovirus-infected cells expressing high levels of shRNA was achieved by sorting cells with high GFP expression using FACS Vantage Cell Sorter (BD Biosciences).

Immunoblotting
Jurkat T cells were treated as indicated, pelleted, and lysed in buffer containing 20 mM Tris (pH 7.5), 40 mM NaCl, 5 mM EDTA, 50 mM NaF, 30 mM sodium pyrophosphate, 1% Triton X-100, and the Protease Inhibitor Cocktail Set V (EMD Biosciences) for 10 min on ice. In some instances, the cells were lysed in buffer containing 300 mM NaCl. Lysates were clarified by centrifugation at 10,000 × g at 4°C. Extracts were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and probed with the indicated Abs and the appropriate secondary Ab conjugated to HRP (DakoCytomation; Jackson ImmunoResearch Laboratories). Monoclonal anti-p52 (C-5), polyclonal anti-lamin B (C-20), monoclonal anti-RelA/p65 (C-20), monoclonal anti-RelB (C-19), monoclonal anti-ICAM-1 (B-6), and polyclonal anti-CD3 (C-19, NLS) were obtained from Santa Cruz Biotechnology, monoclonal anti-κB (6A920) was obtained from Imgenex, monoclonal anti-phospho-IκBα (Ser32/36) (12C2) was obtained from Cell Signaling Technology, and polyclonal anti-Zap70 (MC191) was a gift from...
Dr. P. Leibson (Mayo Clinic College of Medicine). Immunoreactivity was visualized by using an ECL detection system (NEN). In some instances, the level of Rel family proteins was quantified by densitometry analysis and normalized to that of Zap70 or Lamin B signal using the software Quantity One (Bio-Rad).

**Luciferase assay**

A total of 10 × 10⁶ Jurkat cells were transfected with 2 μg of either NF-κB or RE/AP luciferase reporter construct, 0.5 μg of pRL-null (Renilla luciferase; Promega), and 8 μg of control DNA, retroviral construct containing p100ΔN-p100ΔGRR, pSR si-NS, or pSR si-p100, where indicated, by electroporation using a Bio-Rad Gene Pulser Electroporator set at 200 V, 975 μF. Twenty-four hours after transfection, the number of live cells within each sample was determined by trypan blue exclusion. A total of 1 × 10⁶ cells were treated as indicated and luciferase assays were performed in triplicate using the Promega Dual Luciferase Reporter Assay System.

**Nuclear and cytoplasmic extracts**

A total of 5 × 10⁶ Jurkat T cells were treated as indicated before preparation of cytoplasmic and nuclear extracts, as previously described (35). Briefly, cells washed with PBS are gently resuspended in buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 1 mM MgCl₂, 1 mM DTT, 1 mM PMSF, and the Protease Inhibitor Cocktail Set V (EMD Biosciences)), and incubated on ice for 15 min. Nonidet P-40 was added to the lysate for a final concentration of 0.2% and vortexed. Samples were centrifuged at 20,800 × g at 4°C, the cytoplasmic fraction was collected, and the remaining pellet was washed twice in buffer A. Nuclear fractions were obtained by the addition of buffer C (20 mM HEPES (pH 7.9), 0.4 M NaCl, 2 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, and the Protease Inhibitor Cocktail Set V) to the remaining pellet, incubation with agitation at 4°C for 15 min, and centrifugation at 20,800 × g at 4°C. Protein concentrations were determined using Coomassie Plus Protein Assay Reagent (Pierce).

**EMSA**

A total of 3 μg of nuclear extract was incubated at room temperature for 30 min with 1 μg of poly(dI-dC) and binding buffer (5% glycerol, 1 mM EDTA, 50 mM NaCl, and 10 mM Tris-HCl (pH 7.5)). A total of 1 ng of double-stranded, 32P-radioabeled probe corresponding to the binding sites for either NF-κB from the SV40 virus (5′-CTAGTGGGGACCTGCCAC CGGGACTTTCCACTC-3′ (36) or Oct-1 (5′-CTAGTCTGATGC AATACCTAGAA-3′) was added to the binding reaction and incubated for another 30 min. For supershift assays, binding reactions were conducted in the presence of 2 μg of Abs to RelB (Cell Signaling Technology), RelA/p65, cRel, p50, and p52 (Santa Cruz Biotechnology) before the addition of the radiolabeled probe. The DNA-protein complexes were resolved on 5% polyacrylamide gel and subjected to autoradiography. In some instances, the intensity of the NF-κB bands was quantified by densitometry analysis and normalized to the Oct-1 signal using the software Quantity One (Bio-Rad).

**ELISA**

A total of 2 × 10⁵ Jurkat cells in 1 ml were treated as indicated. Culture supernatants were collected and analyzed in triplicate using the Human IL-2 ELISA kit (eBioscience) according to manufacturer’s instructions.

**Immunoprecipitation**

For FLAG immunoprecipitations, 20 × 10⁶ Jurkat T cells stably expressing control protein or FLAG-p100 were lysed in buffer containing 20 mM Tris (pH 7.5), 40 mM NaCl, 5 mM EDTA, 50 mM NaF, 30 mM sodium pyrophosphate, 1% Triton X-100, and the Protease Inhibitor Cocktail Set V for 10 min on ice. Lysates were clarified by centrifugation at 10,000 × g at 4°C and incubated with protein G Sepharose beads (Amersham Biosciences) with agitation for 1 h at 4°C. The beads were removed by centrifugation at 8,000 × g for 30 s and washed five times with lysis buffer. Protein was eluted from beads by incubation with 50 μg/ml FLAG peptide (Sigma-Aldrich) in sample buffer. Lysates were resolved by SDS-PAGE and analyzed by immunoblotting. Endogenous p100 was immunoprecipitated as described previously (37, 38), from 50 × 10⁶ Jurkat cells stimulated with 20 ng/ml PMA and 1 μg/ml ionomycin for 24 h. Briefly, cells were lysed in lysis buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 5 mM EDTA, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1% Nonidet P-40, and the Protease Inhibitor Cocktail Set V for 10 min on ice. Lysates were cleared by centrifugation at 16,000 × g for 10 min. Lysates were incubated with 5 μg of monoclonal anti-p52 Ab (Santa Cruz Biotechnology) for 18 h at 4°C with agitation. Protein G Sepharose beads were added to the mixture and incubated for 1 h at 4°C. Beads were collected by centrifugation, washed five times in lysis buffer, and protein was eluted in sample buffer. Lysates were resolved by SDS-PAGE and analyzed by immunoblotting.

**In vitro kinase assay**

Jurkat T cells were stimulated with anti-CD3 and PMA for the indicated times and lysed in lysis buffer containing 20 mM Tris (pH 7.5), 40 mM NaCl, 5 mM EDTA, 50 mM NaF, 30 mM sodium pyrophosphate, 1% Triton X-100, and the Protease Inhibitor Cocktail Set V. Lysates were incubated with polyclonal anti-IKKα (Santa Cruz Biotechnology) for 2–4 h, and protein G Sepharose beads for an additional hour. The beads were washed twice with lysis buffer and twice with kinase buffer (20 mM HEPES, 20 mM β-glycerophosphate, 10 mM MgCl₂, 100 mM NaCl, 100 μM Na₃VO₄, 2 mM DTT, and the Protease Inhibitor Cocktail). Kinase activity was assayed with kinase buffer, 10 μCi [γ-32P]ATP, 20 μM ATP, and 1 μg GST-InBo (1-54) for 30 min at 30°C. Lysates were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and visualized by autoradiography. Membranes were immunoblotted with anti-IKKβ to ensure equal immunoprecipitation of the kinase complex.

**Real-time quantitative PCR**

Jurkat cells were stimulated with anti-CD3 and PMA for the indicated times and total RNA was obtained using the RNeasy Mini kit (Qiagen). Quantitative real-time PCR was performed by the Mount Sinai Real-Time PCR Shared Research Facility. Briefly, cDNA was generated using 1–5 μg of total RNA, 0.5 μg of dT₁₅, 0.5 mM dNTPs, 50 U Stratascript (Stratagene), and manufacturer-supplied buffer. Real-time PCR was performed using a 1/50 dilution of cDNA, 5 mM MgCl₂, 0.2 mM dNTPs, SYBR Green 1 (Molecular Probes), 2 μM of each primer, 0.25 U PlatinumTaq DNA polymerase (Invitrogen Life Technologies), and manufacturer-supplied buffer. The primers used for real-time PCR are as follows: IκBα sense, 5′-GCCAGCGTCGTACGTTATGATG-3′ and antisense, 5′-AGGAGGGTGATCTCCACCAAA-3′; β-actin: sense, 5′-ACTGGAACGGTGAGGACTG-3′ and antisense, 5′-TGGAATTCCTCGGACGAGACGACTG-3′; p100 sense, 5′-GCCGAGACTTCATCTGACAT-3′ and antisense, 5′-ATGCTCCAGCTAACACTTC-3′; and α-tubulin: sense, 5′-GCTGGACCCACCAAGTTTTGAC-3′ and antisense, 5′-TGAATATCTGAGGGACATGAC-3′.
Results

Induction of p100 expression by TCR activation in T cells

Studies have shown that the activation of the noncanonical NF-κB pathway by the TNFR family and the subsequent processing of NF-κB2/p100 are required for B cell maturation, maintenance, and immune function (14, 39–41). Although the role of NF-κB2/p100 has been extensively studied in B cells, it has not been well-studied in T cells, especially with respect to TCR signaling. In this study, we examined the role of NF-κB2/p100 in TCR signaling. Upon stimulation of human Jurkat T cells with a combination of anti-CD3 (2 μg/ml) plus 20 ng/ml PMA to mimic TCR ligation, the levels of the p100 precursor protein increased in comparison to the unstimulated controls when analyzed by Western blotting with Abs specific to p100/p52. Lysates from Jurkat cells transfected with control or p100 were resolved in parallel as a control for p52 detection. The experiments shown are representatives of at least three experiments that were conducted.

To reduce the level of H chain contamination in samples stimulated with PMA and anti-CD3 as seen above in Fig. 1, we altered the processing of the cells by washing them multiple times before lysis. As a control to monitor the efficiency of the removal of the H chain, the same amount of anti-CD3 was added to one of the samples before removing the cells for washing (Fig. 2A, lane 2), which showed that the H chain contamination has been removed. In addition, the lysis condition was also modified by using a buffer containing 1% Triton X-100 and 300 mM NaCl to extract nuclear proteins, indicated by the presence of lamin B in the lysates. Under these experimental conditions, we also failed to observe a significant increase in the levels of p52 even though p100 up-regulation was observed in response to stimulation with anti-CD3 plus PMA or PMA plus ionomycin (Fig. 2A). As a positive control for the detection of p52 in the Western blots, cellular extracts from Jurkat cells transfected with control or p100 were resolved in parallel as a control for p52 detection. The experiments shown are representatives of at least three experiments that were conducted.

To examine whether the TCR-triggered p100 expression was dependent on the canonical NF-κB pathway by testing the response in a Jurkat T cell mutant lacking the canonical pathway due to a deficiency in IKK-γ/NEMO expression. No significant increase in p100 expression was observed in IKK-γ-deficient Jurkat cells (Fig. 1B), indicating that p100 expression is dependent on the canonical NF-κB-signaling pathway, consistent with that demonstrated in a previous study (42).

The presence of the H chain from the CD3 Ab, which migrates at approximately the same mobility as p52. We next examined whether the TCR-triggered p100 expression was dependent on the canonical NF-κB pathway by testing the response in a Jurkat T cell mutant lacking the canonical pathway due to a deficiency in IKK-γ/NEMO expression. No significant increase in p100 expression was observed in IKK-γ-deficient Jurkat cells (Fig. 1B), indicating that p100 expression is dependent on the canonical NF-κB-signaling pathway, consistent with that demonstrated in a previous study (42).

To reduce the level of H chain contamination in samples stimulated with PMA and anti-CD3 as seen above in Fig. 1, we altered the processing of the cells by washing them multiple times before lysis. As a control to monitor the efficiency of the removal of the H chain, the same amount of anti-CD3 was added to one of the samples before removing the cells for washing (Fig. 2A, lane 2), which showed that the H chain contamination has been removed. In addition, the lysis condition was also modified by using a buffer containing 1% Triton X-100 and 300 mM NaCl to extract nuclear proteins, indicated by the presence of lamin B in the lysates. Under these experimental conditions, we also failed to observe a significant increase in the levels of p52 even though p100 up-regulation was observed in response to stimulation with anti-CD3 plus PMA or PMA plus ionomycin (Fig. 2A). As a positive control for the detection of p52 in the Western blots, cellular extracts from Jurkat

FIGURE 2. p100 expression is induced by TCR stimulation in Jurkat T cells. A, A total of 1 × 10⁶ Jurkat T cells were either left unstimulated, treated with anti-CD3 before lysis, or stimulated with anti-CD3 Ab (2 μg/ml) plus PMA (20 ng/ml) or ionomycin (1 μM) plus PMA (20 ng/ml) for 24 h before lysis with buffer containing 1% Triton X-100 and 300 mM NaCl. As a positive control for the detection of p52, extracts from Jurkat T cells transfected with control or p100-encoding plasmids were resolved in parallel. B, A total of 1 × 10⁶ Jurkat T cells were cocultured with fixed Raji B cells at a ratio of 2:1 (Raji:Jurkat) and 1 μg/ml SED, and incubated for 8, 16, and 24 h. Triton-soluble extracts were analyzed by Western blot analysis using Abs specific to p100/p52. Lysates from Jurkat cells transfected with control or p100 were resolved in parallel as a control for p52 detection. C, A total of 1 × 10⁶ Jurkat T cells cocultured with fixed Raji B cells were stimulated with various concentrations of SED for 24 h. Cellular extracts were analyzed as in A. Lysates from Jurkat cells transfected with control or p100 were resolved in parallel as a control for p52 detection. D, A total of 1 × 10⁶ Jurkat T cells were stimulated with anti-CD3 Ab (2 μg/ml) plus PMA (20 ng/ml), ionomycin (1 μM) plus PMA (20 ng/ml), SED (1 μg/ml) and Raji B cells, or Raji B cells without SED for 24 h. p100/p52 expression was examined in 40 μg of cytoplasmic and nuclear proteins by Western blot. The blot was stripped and reprobed for Zap70 and lamin B to indicate isolation of cytoplasmic and nuclear proteins, respectively. Lysates from Jurkat cells transfected with control or p100 were resolved in parallel as a control for p52 detection.
T cells transfected with a control vector or an expression vector encoding p100 were included in the blots (Fig. 2). Ectopic expression of p100 has been previously shown to result in constitutive processing to p52 (37, 43), which we were also able to detect with the anti-p100/p52 mAb. To confirm the observations obtained with TCR agonists that mimic Ag stimulation, we also used a more physiological system of activating T cells using the superantigen SED presented by fixed Raji B cells. A similar time- and dose-dependent increase in p100 expression with little increase in p52 was observed (Fig. 2, B and C). To exclude the possibility that the p100 has been processed to p52 and translocated to the nucleus, cellular fractionation to isolate the nuclear compartment was performed. As shown in Fig. 2D, there was an increase in p100 expression in the cytoplasmic and nuclear compartments, but very little p52 expression after TCR stimulation (Fig. 2D). In contrast, little p52 was detected in either the cytoplasmic or nuclear compartments.

Finally, the p100 response was also examined in primary human CD4⁺ T cells stimulated with anti-CD3 plus PMA or PMA plus ionomycin for 24 h. A similar up-regulation in p100 expression with little increase in p52 was observed (Fig. 3A). Primary T cells appear to have more detectable p52 compared with Jurkat T cells but the p100:p52 ratio remains substantially in favor of p100. There was donor-to-donor variation in the level of p52 generated upon stimulation of primary T cells and the experiment in Fig. 3A shows one where the highest p52 level was observed. Primary T cells challenged with SEB presented by Raji B cells also demonstrated an increase in p100 expression with minimal generation of p52 (Fig. 3B). These results demonstrate that p100 expression is inducible upon TCR stimulation in human T cells but little processing of p100 to p52 occurs.

**Effect of p100 expression on NF-κB downstream of TCR signaling**

The observation that TCR agonists induce p100 expression without an appreciable increase in p52 level suggests that the noncanonical NF-κB pathway is not activated during T cell activation. However, the question remains as to the role of the elevated p100
observed during this process. In light of the presence of the inhibitory ankyrin repeats in the unprocessed p100 and studies showing that p100 can inhibit NF-κB transcriptional activity and NF-κB-dependent biological processes in osteoclast precursors and dendritic cells (16, 17, 44), we hypothesize that p100 may function as a late-acting negative-feedback molecule to attenuate TCR signaling. To test this hypothesis, we examined the effect of mimicking p100 expression on TCR-induced NF-κB signaling. Because we observed that ectopic expression of p100 resulted in some spontaneous processing to p52, we also tested the effect of the p100ΔGRR mutant, which has a deletion of the glycine-rich region and cannot be processed to p52 (41, 43). Jurkat T cells were transiently transfected with expression vectors encoding NF-κB2/p100, p100ΔGRR, or an irrelevant protein as a negative control, along with an NF-κB-luciferase reporter and Renilla luciferase as a transfection control. Western blot analysis was performed to monitor expression of p100 and p100ΔGRR in each sample (Fig. 4A and C). Stimulation of control-transfected Jurkat T cells with increasing concentrations of SED and Raji B cells or anti-CD3 plus PMA resulted in an increase in NF-κB luciferase reporter activity (Fig. 4B). Transfection of either p100 or p100ΔGRR inhibited NF-κB activity in response to TCR stimulation (Fig. 4B).
FIGURE 6. Inhibition of p100 expression enhances NF-κB transcriptional activity. A and C, Wild-type Jurkat T cells were transiently transfected with pSUPER-retro encoding either a nonsilencing shRNA (si-NS) or shRNA targeted against p100 (si-p100), along with an NF-κB-luciferase reporter (A) or a RE/AP luciferase reporter (C) and pRL-null. Twenty four hours after transfection, 1 × 10^6 cells were lysed and 20 μl of lysate were subject to Western blot analysis using Abs specific to p100/p52 and Zap70. B and D, Jurkat T cells transiently transfected with pSUPER-retro si-NS or si-p100 were either left unstimulated or were stimulated with 25, 50, 75, and 100 ng/ml SED and Raji B cells or anti-CD3 plus PMA for 18 h. The cells were lysed and NF-κB (B) or RE/AP (D) luciferase activity was analyzed in triplicate using the Dual-Luciferase reporter system (Promega). Error bars represent SD. The experiments shown are representative of three experiments that were conducted.

A similar effect was observed with the CD28RE/AP1 reporter derived from the IL-2 promoter (Fig. 4D) that is also responsive to NF-κB signaling (32, 33).

To further examine which step in the NF-κB pathway is inhibited by p100 expression, we performed EMSAs to determine its effect on nuclear NF-κB binding activity. Jurkat T cells stably transfected with NF-κBp50/p100 or an irrelevant control were stimulated with anti-CD3 and PMA, and nuclear extracts were subject to EMSA. In control Jurkat cells, stimulation with anti-CD3 and PMA led to an increase in NF-κB binding activity whereas this response was inhibited in cells expressing p100 (Fig. 5A). A control EMSA performed with a probe containing the Oct-1-binding element demonstrated equivalent extraction of nuclear proteins in all samples (Fig. 5A). Supershift assays conducted on the sample from control-transfected cells stimulated for 8 h confirmed that the complex contains NF-κB factors including RelA and cRel (Fig. 5A). Two anti-p50 Abs were also used in the supershift experiments, only one of which was able to shift the complex indicating the presence of p50 (p50 (2)). No significant shifting was observed with the RelB and p52 Abs. Because these results suggest that ectopic expression of p100 can inhibit NF-κB transcriptional and binding activity, we examined whether the nuclear translocation of the Rel family members is inhibited by p100. Cytoplasmic and nuclear protein extracts from Jurkat T cells stimulated with anti-CD3 and PMA for 4 and 8 h were blotted with Abs specific for RelB, RelA/p65, p50, and cRel (Fig. 5B). Upon TCR stimulation, there is significant nuclear accumulation of RelB, RelA, cRel, and p50 in the control transfected cells. In contrast, nuclear accumulation of Rel proteins was inhibited in the p100-expressing cells (Fig. 5B). These results suggest that p100 attenuates NF-κB transcriptional and binding activity by preventing the Rel proteins from translocating to the nucleus.

To further explore the mechanism by which p100 can inhibit the NF-κB pathway, we examined IKK activity after anti-CD3 plus PMA stimulation. The IKK complex was immunoprecipitated using anti-IKKγ, and kinase activity was measured by an in vitro kinase assay using GST-IκBα (1-54) as the substrate. TCR stimulation resulted in equivalent induction of IKK activity in both control and p100 cells (Fig. 5C). Therefore, p100 does not appear to inhibit activation of the IKK complex. In addition, we also examined the phosphorylation and subsequent degradation of the IκBα inhibitor. TCR activation in control Jurkat T cells induced the phosphorylation of IκBα and its subsequent degradation followed by its resynthesis after 60 min (Fig. 5D). TCR stimulation of the Jurkat cells expressing p100 also resulted in the phosphorylation and degradation of IκBα by 30 min but unlike that in the control cells, no resynthesis of IκBα occurs after 60 min (Fig. 5D). Quantitative real-time PCR also demonstrated an increase in IκBα mRNA levels after TCR activation in control Jurkat T cells (Fig. 5E). However, TCR-mediated IκBα mRNA induction was inhibited in cells expressing p100 (Fig. 5E). These results demonstrated that p100 expression did not affect IKK activity and its phosphorylation of IκBα, indicating the blockade by p100 occurs downstream of IKK. Furthermore, because the IκBα gene is a known target of NF-κB-mediated transcription, the lack of IκBα mRNA induction and resynthesis in the p100-expressing cells supports the hypothesis that p100 inhibits the canonical NF-κB pathway.

Effect of p100 depletion on NF-κB activity

Because ectopically expressed p100 can act as a negative regulator of the canonical NF-κB pathway, we analyzed whether the loss of endogenous p100 resulted in a hyperactive NF-κB response. For these studies, p100 expression in Jurkat T cells was blocked by siRNA-mediated gene silencing using the pSUPER-retro vector. Jurkat T cells were transiently transfected with vectors encoding a nonsilencing shRNA (si-NS) or shRNA targeted against p100 (si-p100), along with an NF-κB-luciferase reporter and Renilla luciferase as a transfection control. Western blot analysis was performed to monitor knockdown of p100 expression upon TCR activation (Fig. 6, A and C). Stimulation of Jurkat T cells with increasing concentrations of SED and Raji B cells or anti-CD3 plus PMA resulted in a dose-dependent increase in NF-κB luciferase reporter activity (Fig. 6B). Knockdown of p100 expression enhanced luciferase activity in response to TCR stimulation, suggesting an increase in NF-κB transcriptional activity. A similar effect of p100 knockdown was observed with the CD28RE/AP1 luciferase reporter (Fig. 6D). These results demonstrate that knockdown of p100 expression enhances NF-κB transcriptional activity upon TCR stimulation consistent with the hypothesis that p100 is a negative regulator of TCR signaling.

To further examine the effect of p100 depletion on NF-κB, we generated Jurkat cells stably transfected with the nonsilencing
control shRNA (si-NS) or shRNA targeting p100 (si-p100). The stimulus-dependent expression of p100 was effectively silenced by si-p100 (Fig. 7A). To examine the effect of p100 depletion on NF-κB nuclear translocation and binding activity, we treated si-NS or si-p100 Jurkat cells with anti-CD3 plus PMA for 16 and 24 h. EMSA were performed as described in Fig. 5A. The intensities of the NF-κB bands were determined by densitometry and normalized to that of the corresponding Oct-1 bands. The induction in NF-κB DNA-binding activity is presented as fold increase over that in the unstimulated control group. The supershift analysis was conducted using nuclear extracts from si-p100 cells stimulated for 24 h and lamin B to indicate isolation of proteins from the cytoplasmic and nuclear compartments, respectively. The intensities of the Rel proteins present in the nuclear extracts were determined by densitometry, normalized to that of the loading controls and are presented as fold increase over that in the unstimulated control sample. D, A total of 1 × 10^6 Jurkat T cells were stimulated with anti-CD3 plus PMA for 5, 15, 30, and 60 min. Total cell extracts were blotted with Abs specific for the phosphorylated form of IkBα and for total IkBα. E, A total of 1 × 10^6 Jurkat T cells were stimulated with anti-CD3 plus PMA for 0, 1, or 2 h. IkBα expression from total RNA was examined by quantitative real-time PCR and normalized to β-actin, α-tubulin, and rps11 expression. The experiments shown are representative of three experiments that were conducted.

FIGURE 7. p100 depletion enhances NF-κB nuclear translocation and binding activity. A, Jurkat T cells were infected with retroviruses generated with pSUPERretro-GFP vector encoding shRNA directed against a nonsilencing target (si-NS) or p100 (si-p100). Transduced cells were selected by cell sorting for high GFP expression. Knockdown of p100 expression was determined by stimulating 1 × 10^6 cells with ionomycin (1 μM) plus PMA (20 ng/ml) for 18 h followed by anti-p100 Western blot analysis. The blot was stripped and probed for Zap70 to ensure equal loading. B, Jurkat T cells stably transfected with si-NS or si-p100 were stimulated with anti-CD3 plus PMA for 16 and 24 h. EMSA were performed as described in Fig. 5A. The intensities of the NF-κB bands were determined by densitometry and normalized to that of the corresponding Oct-1 bands. The induction in NF-κB DNA-binding activity is presented as fold increase over that in the unstimulated control group. The supershift analysis was conducted using nuclear extracts from si-p100 cells stimulated for 24 h. C, Jurkat T cells stably transfected with si-NS or si-p100 were stimulated as indicated. A total of 40 μg of cytoplasmic and nuclear protein were blotted with RelB, RelA/p65, cRel, p50, and p100/p52 Abs. The blots were reprobed for Zap70 and lamin B to indicate isolation of proteins from the cytoplasmic and nuclear compartments, respectively. The intensities of the Rel proteins present in the nuclear extracts were determined by densitometry, normalized to that of the loading controls and are presented as fold increase over that in the unstimulated control sample. D, A total of 1 × 10^6 Jurkat T cells were stimulated with anti-CD3 plus PMA for 5, 15, 30, and 60 min. Total cell extracts were blotted with Abs specific for the phosphorylated form of IkBα and for total IkBα. E, A total of 1 × 10^6 Jurkat T cells were stimulated with anti-CD3 plus PMA for 0, 1, or 2 h. IkBα expression from total RNA was examined by quantitative real-time PCR and normalized to β-actin, α-tubulin, and rps11 expression. The experiments shown are representative of three experiments that were conducted.
FIGURE 8. p100 binds to Rel family members. A. Total cell lysates from Jurkat T cells stably expressing either control protein or FLAG-p100 were immunoprecipitated with anti-FLAG M2 affinity gel. The immunoprecipitates were blotted with Abs for RelA/p65, cRel, RelB, and p50. The blot was stripped and reprobed with anti-FLAG Ab. B. Jurkat T cells were stimulated with PMA (20 ng/ml) plus ionomycin (1 μM) for 24 h. Cell lysates were immunoprecipitated with monoclonal anti-p100/p52 and blotted with RelA/p65, cRel, RelB, and p50 Abs. The membrane was stripped and probed with anti-p100/p52. The experiments shown are representative of three experiments that were conducted.

To further determine the effects of p100 depletion on the NF-κB pathway, we examined the phosphorylation and degradation of IκBα. TCR-induced IκBα phosphorylation, degradation, and subsequent resynthesis were equivalent in both control and si-p100 cells (Fig. 7D). In addition, no difference in TCR-triggered induction of IκBα mRNA was observed between the si-NS and si-p100 cells (Fig. 7E). Taken together, the observations in Fig. 7 demonstrated that a loss of function in p100 did not have an effect on the phosphorylation and degradation of IκBα but it enhanced the translocation of Rel proteins to the nucleus. These results are consistent with the hypothesis that p100 functions as a negative regulator by retaining Rel family members in the cytoplasm.

Mechanism of NF-κB inhibition by NF-κB2/p100

Because the previous experiments demonstrated that p100 did not inhibit IκBα phosphorylation (Fig. 5D) but did inhibit the translocation of Rel proteins to the nucleus (Fig. 5B), the most likely mechanism to explain the inhibitory effect of p100 is that it is binding and thus retaining Rel proteins in the cytoplasm. Previous studies have demonstrated that p100 can bind to RelB, and this complex is retained in the cytoplasm of quiescent B cells (15, 16, 41). Therefore, we stably expressed either FLAG-tagged NF-κB2/p100 or an irrelevant protein in Jurkat T cells and conducted co-immunoprecipitation experiments to test this possibility. Analysis of the anti-FLAG immunoprecipitates by Western blot revealed association of endogenous RelA, RelB, cRel, and p50 with FLAG-p100 (Fig. 8A), consistent with the notion that p100 can bind and retain Rel proteins in the cytoplasm. To confirm that endogenous p100 can also associate with the Rel family members, endogenous p100 was immunoprecipitated with a mAb after stimulation of nontransfected Jurkat T cells with PMA and ionomycin to induce p100 expression. Analysis of the immunoprecipitates also demonstrated association of RelA, RelB, cRel, and p50 with endogenous p100 (Fig. 8B). These results strongly suggest that p100 may inhibit NF-κB activation by binding other Rel family members and retaining them in the cytoplasm.

Effect of p100 expression and depletion on IL-2 production

The hallmark of T cell activation is IL-2 production and subsequent cell proliferation. Because the canonical NF-κB pathway is essential for IL-2 expression (45, 46) and this pathway can be modulated by p100 expression level, we hypothesize that the induction of p100 expression observed in response to TCR stimulation functions to attenuate IL-2 production. To test this hypothesis, the effect of overexpression and knockdown of p100 on TCR-induced IL-2 production was examined. Jurkat T cells stably expressing a control protein, NF-κB2/p100, or p100ΔGRR (Fig. 9A) were stimulated with increasing amounts of SED presented by Raji

FIGURE 9. p100 expression blocks TCR-induced IL-2 production, whereas p100 knockdown enhances IL-2 production. A. Lysates from Jurkat T cells stably expressing control protein, p100, or p100ΔGRR were subjected to Western blot analysis using Abs specific to p100/p52 and zap70. B. A total of 2 × 10⁵ Jurkat T cells stably expressing either control protein, p100, or p100ΔGRR were cocultured with Raji B cells and stimulated with varying concentrations of SED for 18 h. IL-2 concentrations in the culture supernatants were measured in triplicate by ELISA. Error bars, SD. C. A total of 2 × 10⁵ Jurkat T cells stably expressing control shRNA (si-NS) or p100 shRNA (si-p100) were cultured with Raji B cells and stimulated with varying concentrations of SED for 18 h. IL-2 was measured as in B. The experiments shown are representative of three experiments that were conducted.
The results in this report suggest that p100 expression serves to inhibit TCR-mediated NF-κB signaling. A prior study by Li et al. (37) reported the activation of NIK and IKKa activity, which are principal components of the noncanonical NF-κB pathway, in response to TCR/CD28 cross-linking within the first 30 min (37). Interestingly, despite the activation of NIK and IKKa, very little processing of p100 to p52 was also observed and the molecular basis for this observation remains unclear. It is a possibility that these two kinases are no longer active by the time p100 levels are elevated, i.e., after extended TCR ligation. It is also interesting that we observed some processing to p52 when p100 is ectopically expressed, but very little processing with the p100 that is induced by TCR agonists. One trivial explanation for the difference is that the level of p100 obtained with transfection is significantly higher than the level of endogenous p100 obtained by stimulation. The high level of p100 achieved by ectopic expression is sufficient to trigger processing to p52 as has been demonstrated by others (37, 43). Alternatively, there may be suppressive mechanisms that prevent p100 from being processed during T cell activation. In the absence of direct evidence, this remains a speculation.

NIK has also been previously implicated in TCR signaling based on the observation that T cells from alymphoplasia (aly) mice, which has a mutation in NIK, exhibited defects in NF-κB signaling and IL-2 production upon TCR activation (18, 49). An alternative explanation for the phenotype of the aly T cells based on our study is that the signaling defect may not be due to a direct effect of the mutation on the TCR-signaling pathway, but rather may be an indirect effect caused by an accumulation of p100 induced by the TCR or other receptors that activate the canonical NF-κB pathway. Therefore, results obtained from experiments conducted with aly and NIK-deficient cells should be interpreted to distinguish between 1) a direct effect of the NIK deficiency, 2) an indirect effect due to p100 accumulation, and 3) indirect effects on yet-to-be-determined NIK targets. In line with this, Ishimaru et al. (18) recently observed that memory T cells from aly mice have reduced TCR-induced IL-2 expression and this correlated with an accumulation of unprocessed p100 leading to the suggestion that p100 is a negative regulator (18). Alternatively, Sanchez-Valdepenas et al. (50) reported that NIK phosphorylates the transactivation domain of cRel to activate its transcriptional activity and therefore, the phenotype of the aly T cells may also be due to the lack of cRel phosphorylation (50). Another factor to consider with the aly T cells is the possibility that NIK may phosphorylate other substrates that have not been characterized and the phenotype of the aly T cells may relate to the lack of phosphorylation of these substrates. Because of these different possibilities, the use of NIK-deficient cells does not directly address the role of NF-κB2/p100 in a particular pathway. Our results now provide direct evidence to support the notion that p100 functions as a negative regulator during TCR signaling.

In addition to the current study suggesting a negative regulatory role for p100 in T cell activation, a negative regulatory role for p100 in other biological processes involving NF-κB signaling has also been proposed for osteoclastogenesis (17) and dendritic cell maturation (44). One mechanism for NF-κB inhibition by p100 may be found in its structure. The presence of the IkB-like ankyrin repeats at its C-terminal end allows p100 to bind to other members in the NF-κB family to inhibit their translocation to the nucleus (10, 16). In fact, p100 was shown to be bound to RelB in the cytoplasm of resting B cells and upon ligation of CD40, p100 is cleaved allowing the p52:RelB dimer to be translocated to the nucleus (41). Studies in mouse CD4+ T cells have shown that p52 and p100 associated with RelB, RelA, and p50 (18). Similarly, we found that p100 also binds to RelB in Jurkat T cells, as well as to RelA, cRel, and p50. Due to the limited processing to p52, this

![FIGURE 10. Model for the negative regulation of TCR signaling by NF-κB2/p100. TCR activation results in the phosphorylation of IkBα by the IKK complex, leading to its degradation. Free RelA/p50 dimers translocate to the nucleus where they up-regulate IkBα expression within the first hour and p100 expression after several hours of TCR stimulation. This model proposes that a negative-feedback mechanism involving IkBα occurs during the early stages, which is followed by another negative-feedback mechanism involving p100 during the later stages of T cell activation.](Image)
binding will lead to the retention of these transcription factors in the cytoplasm. In addition to similarities in structure, the negative regulation of the canonical NF-κB pathway by p100 may be similar to that of IkBα. As described earlier, induction of IkBα by the canonical NF-κB pathway provides a negative feedback mechanism leading to the inhibition of relA/p50 dimers (29). Like IkBα, the expression of p100 is also dependent on the canonical NF-κB pathway as shown here and by others (42). However, whereas the re-expression of IkBα occurs within minutes after its degradation, the expression of p100 occurs hours after T cell activation. This would suggest that during T cell activation, a negative feedback mechanism involving IkBα occurs during the early stages of TCR signaling, while a negative feedback mechanism involving p100 may occur during the later stages of TCR signaling (Fig. 6). Consistent with our model, knockdown of p100 did not have any effect on the induction of an immediate early gene such as IkBα during the first hour of stimulation (Fig. 7), most likely because the p100 level is not up-regulated at that time point (Fig. 1). In contrast, knockdown of p100 did have an enhancing effect on IL-2 production (Fig. 9), most likely because IL-2 gene expression occurs with slower kinetics compared with IkBα. It is unclear at this point why p100 expression is considerably slower than that of IkBα even though both are dependent on the canonical NF-κB pathway. It is possible that p100 expression is dependent on a factor that has to be induced or requires an additional signaling pathway that is activated with slower kinetics.

In summary, little is currently known about how TCR-induced NF-κB signaling is negatively regulated. This negative regulation is crucial in controlling the duration of TCR signaling and preventing abnormal lymphocyte activation and proliferation (27). This study demonstrates that NF-κB2/p100 can act as a late-acting negative feedback molecule to attenuate TCR-mediated NF-κB signaling. Understanding the mechanisms by which the TCR-dependent NF-κB-signaling pathway is negatively regulated will lead to a greater understanding of the control of TCR signaling and perhaps will identify targets for therapeutic intervention and modulation of T cell function.

Acknowledgments

We thank Drs. Warner Greene, Shao-Cong Sun, Arthur Weiss, Paul Leibon, Benjamin Chen, Huabao Xiong, and Lloyd Mayer for generously sharing reagents. We also thank the Mount Sinai Real-Time PCR Shared Research Facility for help with quantitative real-time PCR.

Disclosures

The authors have no financial conflict of interest.

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